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(57) Abstract

An assay to simultaneously detect the presence of HIV-1 antibody and/or HIV-2 antibody in a test sample is disclosed. The analytes are captured on either the same or different solid phases, and the presence of the analytes is determined by detecting a signal generated by using a cocktail of synthetic and recombinant antigen-containing indicator reagents.

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HIV IMMUNOASSAY UTILIZING RECOMBINANT PROTEIN AND SYNTHETIC PEPTIDE REAGENTS

This application is a continuation-in-part of U.S.Serial No. 07/866,380, filed April 9, 1992, which is a continuation-in-part application of U.S.Ser. No.07/787,710 filed November 4, 1991, which is a continuation application of U.S.Ser. No.. 07/361,733 filed June 2, 1989 (abandoned), which is a continuation-in-part application of U.S.Ser. No. 07/320,882 filed March 9, 1989 (abandoned), which is a continuation application of U.S.Ser. No. 07/020,282 filed February 27, 1987 (abandoned) which is a continuation-in-part application of U.S.Ser. No. 06/811,240 filed December 20, 1985 (abandoned), all of which enjoy common ownership and are incorporated herein in reference.

Field of the Invention

This invention relates to human immunodeficiency virus (HIV) and immunoassays. In particular, this invention relates to an immunoassay and reagents for the simultaneous detection of HIV-1 antigens and/or HIV-1 antibodies and HIV-2 antibodies in a test sample.

Background of the Invention

Acquired immunodeficiency syndrome (AIDS) is a disorder of the immune system associated with opportunistic infections and/or neoplasms which has reached epidemic proportions in the United States as well as in Europe and in central Africa. The epidemiological data suggest that AIDS is caused by at least two types of human immunodeficiency viruses, collectively designated as HIV. HIV type 1 (HIV-1) has been isolated from patients with AIDS and AIDS-related complex (ARC), and from healthy persons at high risk for AIDS. See, for example, F. Barre-Sinoussi et al., Science 220:868-871 91983); M. Popovic et al., Science 224:497-500 (1984); and R. C. Gallo et al., Science 224:500-503 (1984). HIV-1 is transmitted by sexual contact, exposure to blood and certain blood products, or from an infected mother to her fetus or child. P. Piot et al., Science 239:573-579 (1988). The prevalence of HIV-1

antibodies in AIDS and ARC patients and persons at risk is high, and the virus can be isolated from nearly 90% of all seropositive individuals. See, for example, M. G. Sarngadharan et al., Science 224:506-508 (1984); and D. Gallo et al., J. Clin. Micro. 25:1291-1294 (1987).

In 1986 a second human immunodeficiency virus, HIV-2, was isolated from patients with AIDS in west Africa. F. Clavel et al., Science 233:343-346 (1986). HIV-2 infections also have been identified in individuals from several countries outside of west Africa. See, for example, A. G. Saimot et al., Lancet i:688 (1987); M.A. Rey et al., Lancet i:388-389 (1986); A. Werner et al., Lancet i:868-869 (1987); G. Brucker et al., AIDS 2:141 (1988); and K. Marquart et al., AIDS 2:141 (1988). Although at the present time HIV-2 appears to be endemic only in west Africa, it appears likely that, based on the experience with HIV-1, HIV-2 will spread to other parts of the world.

HIV-2 virus is similar to HIV-1 virus in its morphology, cell tropism, interaction with the CD4 cellular receptor, in vitro cytopathic effect on CD4 cells, overall genomic structure and its ability to cause AIDS. F. Clavel, AIDS 1:135-140 (1987). However, HIV-2 differs from HIV-1 in several respects. See F. Clavel, Ibid and R.A. Weiss et al., AIDS 2:95-100 (1988).

Serological tests indicate that HIV-1 and HIV-2 share multiple common epitopes in their core antigens, although their envelope glycoproteins are much less cross-reactive. F. Clavel, supra. This limited cross-reactivity of the envelope antigens may explain the failure of most currently-available serological assays for HIV-1 to react with certain sera from individuals with antibody to HIV-2. F. Denis et al., J. Clin. Micro. 26:1000-1004 (1988). A commercially available assay for HIV-1/HIV-2 antibody, recently available from Abbott Laboratories, Abbott Park, IL 60064, designated as the Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA, uses recombinant antigens corresponding to the two viral proteins, HIV-1 envelope and HIV-2 envelope. The use of these recombinant antigens allows for the improved detection of anti-HIV-1 and/or anti-HIV-2 containing test samples, while minimizing non-specific reactions largely due to cross reactions with whole virus or

viral lysate. The use of at least one recombinant HIV protein to detect HIV antibody in a test sample with the use of labeled recombinant HIV antigens is described in the parent patent applications previously incorporated herein by reference.

Immunoassays have been previously described to detect the presence of antibodies to the HIV virus in human sera used an enzyme linked immunosorbent assay (ELISA) method employing as the antigen reagent inactivated whole virus obtained from a cell line capable of virus replication . Subsequent immunoassays for HIV describe the use therein of polypeptide sequences obtained by recombinant DNA methodology. See Cabradilla, et al., Bio/Technology, 4: 128-133 (1985). However, such previously described immunoassays lack sensitivity and specificity which could permit test samples, such as blood products containing virus to escape detection and thereby potentially result in the infection of those patients receiving, for example blood products. The lack of specificity (i.e., false positives) in such immunoassays is often due to nonspecific binding of immunoglobulins to cellular protein in the viral lysates, or in the case of recombinant antigens, lack of specificity may be caused by shared epitopes with viruses unrelated to AIDS. For example, Gallaher, Cell, 50: 327-328 (1987) has reported that a region of HIV-1 gp41 shares an antigenic region with the respiratory syncytial virus and with the measles virus F1 glycoprotein. Thus, even highly purified recombinant HIV polypeptides could potentially be responsible for false positives. In either case, such false positives could result in the misdiagnosis of AIDS.

Based on the nucleotide analysis of the viral genome the HIV genomic RNA encodes (beginning at the 5' end):

(i) a gag gene extending between nucleotides 310 to 1869 and encoding for the internal structure core or nucleocapsid proteins including p24, the most antigenic core protein;

(ii) a pol gene extending between nucleotides 1,629 to 4,673 and encoding for the enzyme, reverse transcriptase; and

(iii) an env gene extending between nucleotides 5,781 to 8,369 and encoding for the envelope glycoprotein including gp41,

284 (1985).

One of the challenges faced by today's medical community is the protection of blood products from contamination by HIV, which has been found in blood products (as well as other human body fluids), and which reportedly has been transmitted in the blood supply. Several assays are available to date, including the assay described in U.S. Patent No. 4,520,113 to Gallo et al.

Also, other assays which can detect HIV antigen or HIV antibody are known. Such assays include the anti-HIV-1/HIV-2 assay described hereinabove and disclosed in the previously referenced patent application incorporated herein by reference, as well as those taught by U.S. Patent No. 4,748,110 to D. Paul, U.S. Patent No. 4,983,529 to J. Stewart et al., and U.S. Patent Application Serial No. 07/204,798., all of which enjoy common ownership and are incorporated herein by reference. However, all known federally-approved assays for detection of HIV antigen analyte or HIV antibody analyte are only capable of separately detecting either HIV antigen analyte or HIV antibody analyte in a test sample. No known commercially available, federally-approved assay is available for detection of both HIV antigen analyte and/or HIV antibody analyte in a single assay using a test sample.

The detection of more than one analyte in a test sample usually involves the separate detection of each analyte in a separate assay. Such detection methods have been preferred since they allow for stringent quality assurance determinations to be performed for each analyte to be tested.

Advances in medicine have brought a recognition of new markers for many diseases and clinical conditions, along with the demand for clinical tests for these markers. Laboratories are faced with the problem of providing increasing amounts of tests in a timely manner while attempting to keep costs down. For example, the testing requirements of blood banks have increased dramatically due to the addition of Human T-Leukemia Virus Type 1 (HTLV-1), HIV and Hepatitis C Virus (HCV) to the panel of agents tested in these laboratories on donor blood for the presence of or exposure to these agents.

One possible solution to reducing the laboratory workload brought about as a result of testing requirements, especially in blood banks, is to find ways to combine assays. However, combining assays without compromising their individual performance standards is difficult and more importantly, the problems involved in manufacturing and quality control, can be insurmountable.

Assays to simultaneously detect more than one analyte in a test sample would be advantageous since the time involved in detecting more than one analyte in the test sample would diminish considerably, and the cost of each assay would be lowered since less technical time, reagents, and equipment would be required to perform such an assay.

For example, U.S. Patent No. 4,315,907 to Fridlender et al. teaches a heterogeneous specific binding assay system wherein separation of bound-species from a free-species form of the labeled reagent occurs.

U.S. Patent No. 4,378,344 and EP 027008 to Zahradnik et al. teach a solid phase device for determining the presence of each analyte comprising a receptacle and an insert wherein the presence of each analyte is determined by the claimed assay method.

Great Britain Patent No. 2188418 teaches an assay tray assembly having reaction wells each with openings in the top surface from which a projection is extended and wherein the inner surface of each well sidewall and the outer surface of each projection may be incubated simultaneously for detecting two or more specific substances present in a specimen which has been introduced into the reaction wells.

EPA No. 0 351 248 to applicant IDEXX Corporation discloses a simultaneous immunoassay for feline viruses or HIV in which an antigen and/or antibody member of a single binding pair are detectable. Also, U. S. Patent No. 5,039,604 to Papsidero teaches an immunoassay which simultaneously detects two HTLV or HIV antibodies by adding two different antigens and then a single labeled antibody which is reactive with both antigens. In addition, U.S. Patent No. 4,870,003 to Kortright et al. discloses a solid phase

single binding pair utilizing an antigen "spike" of inactivated antigen.

Also, the detection of one or more analytes using two or more solid phases is the subject matter of co-pending U. S. Patent Application Serial No. 574,821, which enjoys common ownership and is incorporated herein by reference.

Factors which have been identified for the successfuldevelopment of simultaneous assays are that the two assays to be performed simultaneously must have the same sample volumes, identical incubation times and identical cut-off calculations. Such a simultaneous assay also should be capable of being separately quality controlled for each analyte, both at the manufacturer and at the laboratory using the assay, to ensure the sensitivity, specificity and reproducibility of the immunoassay.

It therefore would be advantageous to provide an assay wherein the presence of more than one HIV analyte, i.e., HIV-1 and/or HIV-2 antibodies or antigens could be simultaneously detected, yet each separate analyte to be detected could be individually quality controlled. Such an assay would be an improvement over other known assays since the simultaneous determinations of the presence of either HIV antigen analyte and/or HIV antibody analyte would be performed in one well, separation of solid phase components would not be required if more than one solid phase was utilized, and the assay could be quality controlled for individual analytes which were to be detected in the simultaneous assay.

Furthermore, it would be advantageous to provide a diagnostic assay employing synthetic HIV antigens having unique and highly conserved epitopes of the HIV virus. Such synthetic HIV antigens are beneficial because of the relative ease and lower cost with which they can be prepared, and more importantly, because of the reduced risk of obtaining false positives due to impurities or presence of shared epitopes with viral proteins not related to AIDS.

Summary of the Invention

According to the present invention, an immunoassay for HIV-1 and/or HIV-2 is provided comprising the steps of contacting a test sample with recombinant antigens (hereinafter "capture reagents") of (a) HIV-2 gp36 env , (b) HIV-1 gp41 env , and (c) HIV-1 p24 gag immobilized on one or more solid materials to form a first mixture. The first mixture is incubated for a time and under conditions sufficient to form HIV env antibody/ recombinant antigen complexes and HIV gag antibody/recombinant antigen complexes, and the resulting complexes are contacted with (a) an improved indicator reagent comprising a synthetic antigenic site-directed HIV-2 env antigen labelled with a signal generating compound, (b) a recombinant HIV-1 env antigen labeled with a signal generating compound, and (c) a recombinant HIV gag antigen labeled with a signal generating compound to form a second mixture. The second mixture is incubated for a time and under conditions sufficient to form HIVantibody/ capture reagent/indicator reagent complexes. The presence of HIV-1 env antibodies, HIV-2 env antibodies, and/or HIV gag antibodies in the test sample is determined by detecting the total signal generated by the complexes.

Improved senstivity of the assay of the present invention was found when at least one indicator reagent comprised a synthetic site-directed HIV env antigen having an immunoreactive specificity characteristic of one immunodominant region of gp36 of HIV-2. In particular, the present invention unexpectedly and surprisingly found that the inclusion of a HIV-2 gp36 synthetic peptide in the assay of the present invention significantly reduced the frequency of HIV-2 false positives as compared to HIV immunoassays emplying only recombinant antigen.

Test kits for performing the assays of the present invention also are provided.

Detailed Description of the Invention

Assay Formats

Tthe detection of HIV antigen analyte and/or HIV-1 and HIV-2 antibody analytes in a test sample of the present invention can be performed according to various homogeneous and heterogenous assay formats known in the art where the various reagent additions as described above can be perforemed simultaneously or sequentially.. The assay of the present invention is preferably in an immunoassay format, although the present invention is not limited to immunoreactive assays. For example, any assay utilizing specific binding members can be performed. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding member, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments; antibodies and antibody fragments, both monoclonal and polyclonal; and complexes thereof, including those formed by recombinant DNA methods.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as, an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. As a member of a specific binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of

intrinsic factor protein in the capture and/or indicator reagents for the determination of vitamin B_{12}, or the use of a lectin in the capture and/or indicator reagents for the determination of a carbohydrate. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances.

The test sample can be a mammalian biological fluid such as whole blood or whole blood components including red blood cells, white blood cells including lymphocyte or lymphocyte subset preparations, platelets, serum and plasma; ascites; saliva; stools; cerebrospinal fluid; urine; sputum; trachael aspirates and other constituents of the body which may contain or be suspected of containing the analyte(s) of interest. The test sample also can be a culture fluid supernatant, or a suspension of cultured cells. Mammals whose body fluids can be assayed for HIV antigen analyte or HIV antibody analyte according to the present invention include humans and primates, as well as other mammals who are suspected of containing these analytes of interest. It also is contemplated that non-biological fluid samples can be utilized.

The methods of the present invention are advantageously used in solid phase heterogeneous binding assays which include both sandwich and competitive assay methods. Heterogeneous binding assay techniques involve the use of a solid phase material to which a member of the binding reaction becomes bound. Prior to detecting the label which indicates the presence or amount of analyte in the test sample, the immobilized reaction component is separated from excess sample and assay reagents by removing the solid phase from the reaction mixture.

In a solid phase sandwich assay, a capture reagent as defined below typically involves a capture binding member which has been bound to a solid phase material. For example, the specific binding member can be an immobilized antibody which will bind to an antigen-analyte in the test sample, or the specific binding member can be an immobilized antigen which will bind to an antibody-analyte in the test sample. The capture reagent is

to an indicator reagent comprising a second specific binding member that has been labeled; for example, a labeled anti-analyte antibody or labeled antigen. The reagents can be mixed simultaneously or added sequentially, either singly or in combination. A binding reaction results in the formation of a capture reagent/analyte/indicator reagent complex. The assay can also comprise the step of separating the resultant complex from the excess reagents and test sample. The complex retained on the solid phase material is detected by examining the solid phase for the indicator reagent. If analyte is present in the sample, then label will be present on the solid phase material. The amount of label which becomes associated with the solid phase is directly proportional to the amount of analyte in the sample.

The assays of the present invention can be carried out using any of the sandwich assay formats, including the forward, reverse and simultaneous techniques. Typically, a forward assay involves the contact of the test sample to the capture reagent followed by an incubation period which is in turn followed by the addition of the indicator reagent. A reverse assay involves the addition of the indicator reagent to the test sample followed by the addition of the capture reagent after an incubation period. A simultaneous assay involves a single incubation step as the capture reagent and indicator reagent are both contacted to the test sample at the same time.
member.

Competitive assays can also be carried out using the antigens of the present invention. In a solid phase competitive assay, the capture reagent again typically involves a capture binding member which has been affixed to a solid phase material and which is contacted with both test sample and an indicator reagent. The indicator reagent, however, can be formed from an analyte or analyte-analog which has been conjugated with a label. A binding reaction occurs and results in the formation of complexes of (1) immobilized capture reagent/analyte complex and (2) immobilized capture reagent/indicator reagent complex. Alternatively, the immobilized specific binding member can be an

analyte or analyte-analog with which the test sample analyte competes for binding to the indicator reagent. In the competitive assay, the amount of label which becomes associated with the solid phase is inversely related to the amount of analyte in the sample. Thus, a positive test sample will generate a decrease in signal.

In these binding assays, the presence or amount of the analyte in the test sample is usually determined by detecting the presence or amount of the label which has become associated with the solid phase, although free or unbound indicator reagent may also be detected. In the competitive assay, the more analyte present in the test sample the lower the amount of label present on the solid phase. In the sandwich assay, the more analyte present in the sample the greater the amount of label present on the solid phase.

Indicator Reagents

The indicator reagents of the present invention comprise a specific binding member of each analyte conjugated to a signal generating compound (label). Each indicator reagent produces a detectable signal at a level relative to the amount of the analyte in the test sample. In a preferred embodiment, each indicator reagent, while comprising a specific binding member of a different analyte, is conjugated to the same signal generating compound, which is capable of generating a detectable signal. In general, the indicator reagent is detected or measured after it is captured by the capture reagent. In the present invention, the total signal generated by the indicator reagent(s) indicates the presence of one or more of the analytes in the test sample. It is contemplated that different signal generating compounds can be utilized in the practice of the present invention. Thus, for example, different fluorescent compounds could be utilized as the signal generating compounds, one for each indicator reagent, and detection could be determined by reading at different wavelengths. Or, a short-lived chemiluminescent compound such as an acridinium or phenanthrindium compound and a long-lived chemiluminescent compound such as a dioxetane can be utilized to generate signals at different times for different analytes. Methods which detail the

use of two or more chemiluminescent compounds which are capable of generating signals at different times are the subject matter of co-pending patent application U.S. Serial No. 636,038, which enjoys common ownership and is incorporated herein by reference. Acridinium and phenanthridinium compounds are described in co-pending U.S. patent application Serial No. 07/271,763 filed June 23, 1989, which enjoys common ownership and is incorporated herein by reference.

In addition to being either an antigen or an antibody member of a specific binding pair, the specific binding member of the indicator reagent can be a member of any specific binding pair, including either biotin or avidin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor or an enzyme, an enzyme inhibitor or an enzyme, and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to the analyte as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay. If an antibody is used, it can be a monoclonal antibody, a polyclonal antibody, an antibody fragment, a recombinant antibody, a mixture thereof, or a mixture of an antibody and other specific binding members. The details of the preparation of such antibodies and their suitability for use as specific binding members are well known to those in the art.

In a preferred embodiment, the antigen of the indicator reagent is a synthetically- or recombinantly-produced antigen capable of binding to the immunodominant regions of the core and env proteins of HIV-1 and the env region of HIV-2. In one embodiment, the antigen used in the HIV-2 indicator reagent is a synthetically produced peptide corresponding to the immunodominant region of gp36 of HIV-2 and is characterized by its ability to immunoreact with antibodies induced by HIV-2. The synthetic peptide is from between about 10 amino acids to about 50 amino acids in length and includes the amino acid residue sequence -CAFRQVC-. Preferred synthetic peptides include, but are not limited to:

(a) Lys-Asp-Gln-Ala-Gln-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr;

(b) Arg-Val-thr-Ala-Ile-Glu-Lys-Tyr-LeuLys-Asp-Gln-Ala-Gln-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr; and

(c) Val-thr-Ala-Ile-Glu-Lys-Tyr-Leu Glu-Asp-Gln-Ala-Arg-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys.

It should be understood that a peptide of the present invention need not be identical to the specific amino acid sequences presented above so long as the peptide contains CAFRQVC or homologs thereof and are able to bind with antibodies induced by HIV-2. Moreover, the synthetic peptide of the present invention can be subject to various changes, such as insertions and deletions, and substitutions of one amino acid for another, either conservative or nonconservative in nature.

The peptides of this invention are preferably prepared using conventional solid phase synthesis. However, other well known methods of peptide synthesis may also be used. The resin support is any suitable resin conventionally employed in the art for solid phase preparation of peptides, preferably p-methylbenzyloxyalcohol polystyrene and p-methylbenzydrlamine resin. Following the coupling of the first protected amino acid to the resin support, the amino protecting group is removed by standard methods conventionally employed in the art of solid phase peptide synthesis. After removal of the amino protecting group, remaining α-amino protected and, if necessary, side chain protected amino acids are coupled, sequentially, in the desired order to obtain the product.

The selection of an appropriate coupling reagent follows established art. For instance, suitable coupling reagents are N,N'-diisopropylcarbodiimide or N,N'-dicyclohexylcarboiimide (DCC) either alone or preferably in the presence of 1-hydroxybenzotriazole. Another useful coupling procedure makes use of performed symmetrical anhydrides of protected amino acids.

Cyclic peptides of this invention are prepared by the direct oxidative conversion of protected or unprotected SH-groups to a

disulfide bond following techniques generally known in the art of peptide synthesis. The preferred method involves the direct oxidation of free SH-groups with potassium ferricyanide. Such cyclic peptides are believed to assume more rigid conformation with may favor binding to HIV antibodies.

The label of the indicator reagent is capable of generating a measurable signal detectable by external means. The labels contemplated include, but are not intended to be limited to chromogens; catalysts such as enzymes for example, horseradish peroxidase, alkaline phosphatase, and B-galactosidase; luminescent compounds such as fluorescein and rhodamine; chemiluminescent compounds such as acridinium compounds, phenanthridinium compounds and dioxetane compounds; radioactive elements; and direct visual labels. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances. A variety of different indicator reagents can be formed by varying either the label or the specific binding member.

Capture Reagents

The capture reagents of the present invention comprise a specific binding member for each of the analytes of interest which are attached to at least one solid phase and which are unlabeled. Although the capture reagent is specific for the analyte as in a sandwich assay, it can be specific for indicator reagent or analyte in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample. This attachment can be achieved, for example, by coating the specific binding member onto the solid phases by absorption or covalent coupling. Coating methods, and other known means of attachment, are known to those in the art.

The specific binding member of the capture reagent can be any molecule capable of specifically binding with another

molecule. The specifi: inding member of the capture reagent can be an immunoreactive compound such as an antibody, antigen, or antibody/antigen complex. If an antibody is used, it can be a monoclonal antibody, a polyclonal antibody, an antibody fragment, a recombinant antibody, a mixture thereof, or a mixture of an antibody and other specific binding members.

The "solid phase" is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads and microparticles, membranes, plastic tubes, walls of wells of reaction trays, glass or silicon chips and tanned sheep red blood cells are all suitable examples. Suitable methods for immobilizing capture reagents on solid phases include ionic, hydrophobic, covalent interactions, and the like. In one example of the present invention, 60-well polystyrene reaction trays and 1/4 inch polystyrene beads are utilized, while in another example, a 96-well reaction tray is the only solid phase utilized. It is contemplated that all solid phases be present during the quantitation of signal, thus eliminating the need to separate solid phases for detection of signal.

A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead,

the solid phase also can comprise any suitable porous material with sufficient porosity to allow access by detection antibodies and a suitable surface affinity to bind antigens. Microporous structures are generally preferred, but materials with gel structure in the hydrated state may be used as well. Such useful solid supports include but are not intended to be limited to natural polymeric carbohydrates and their synthetically modified, cross-linked or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums, cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers which may be prepared with suitably porous structures, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; porous inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer. All of these materials may be used in suitable shapes, such as films, sheets, or plates, or they may be coated onto or bonded or laminated to appropriate inert carriers, such as paper, glass, plastic films, or fabrics.

The porous structure of nitrocellulose has excellent absorption and adsorption qualities for a wide variety of reagents including monoclonal antibodies. Nylon also possesses similar characteristics and also is suitable.

It is contemplated that such porous solid supports described hereinabove are preferably in the form of sheets of thickness from about 0.01 to 0.5 mm, preferably about 0.1 mm. The pore size may vary within wide limits, and is preferably from about 0.025 to 15 microns, especially from about 0.15 to 15 microns. The surfaces of such supports may be activated by chemical processes which cause covalent linkage of the antigen or antibody to the support. The irreversible binding of the antigen or antibody is obtained, however, in general, by adsorption on the porous material by poorly understood hydrophobic forces.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the test sample.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly to the material or onto microparticles which then are retained by a solid phase support material. Alternatively, microparticles can serve as the solid phase, by being retained in a column or being suspended in the mixture of soluble reagents and test sample, or the particles themselves can be retained and immobilized by a solid phase support material. By "retained and immobilized" is meant that the particles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. The particles can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. The size of the particles is not critical, although it is preferred that the average diameter of the particles be smaller than the average pore size of the support material being

used. Thus, embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in co-pending U. S. Patent Application Serial No. 150,278 corresponding to EP Publication No. 0326100, and U. S. Patent Application Serial No. 375,029 (EP Publication No. 0406473), which enjoy common ownership and are incorporated herein by reference, can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged poly-anion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in co-pending U.S. Patent Application Serial No. 921,979 corresponding to EPO Publication No. 0 273,115, which enjoys common ownership and which is incorporated herein by reference.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U. S. Patent Application 425,651 and U. S. Patent No. 5,089,424, which correspond to published EPO applications Nos. EP 0 425 633 and EP 0 424 634, respectively, and U.S. Patent No. 5,006,309 all of which enjoy common ownership and are incorporated herein by reference. Such systems also include U.S. Patent Application Serial No. 07/859,218 filed March 27, 1992, which enjoys common ownership and is incorporated herein by reference.

In the practice of one embodiment of the present invention, a test sample suspected of containing any of the HIV antigen analyte or HIV antibody analytes of interest is simultaneously contacted with a solid phase to which a first specific binding member of a first analyte is attached, and a solid phase to which a first specific

binding member of a second analyte has been attached, thereby forming a mixture. The specific binding members serve as capture reagents to bind the analyte(s) to the solid phases. If the specific binding member is an immunoreactant, it can be an antibody, antigen, or complex thereof, specific for each analyte of interest. If the specific binding member is an antibody, it can be a monoclonal or polyclonal antibody, an antibody fragment, a recombinant antibody, as well as a mixture thereof, or a mixture of an antibody and other specific binding members. This mixture is incubated for a time and under conditions sufficient for a binding reaction to occur and which incubation results in the formation of capture reagent/first analyte complexes of the first analyte if it is present in the test sample, and/or the formation of capture reagent/second analyte complexes of the second analyte if it is present in the test sample.

Then, an indicator reagent for each analyte is contacted with the complexes. The indicator reagent for the first analyte comprises a specific binding member of the first analyte of interest which has been labeled with a signal generating compound. The indicator reagent for the second analyte comprises a specific binding member of the second analyte of interest which has been labeled with the same signal generating compound as the indicator reagent for the first analyte, thereby forming a second mixture. This second mixture is incubated for a time and under conditions sufficient to form capture reagent/first analyte/indicator reagent complexes and/or capture reagent/second analyte/indicator reagent complexes. The presence of either analyte is determined by detecting the signal generated in connection with the complexes formed on the solid phase as an indication of the presence of one or more analytes in the test sample. If the indicator employs an enzyme as the signal generating compound (label), then the signal can be detected visually or measured spectrophotometrically. Or, the label can be detected by the measurement of fluorescence, chemiluminescence, radioactive energy emission, etc., depending on the label used to generate the signal.

The capture reagents can be attached to the same solid phase, or can be attached to different solid phases. It is contemplated that all capture reagents could be attached to the same solid phase, or that each capture reagent could be attached to a separate solid phase, or that combination of capture reagents could be attached to separate solid phases. For example, if microparticles were the solid phase of choice, then separate microparticles could have at least one capture reagent(s) attached to it. A mixture of microparticles (solid phases) could be used to capture the various analytes which may be present in the test sample by using the mixture of microparticles. It is contemplated that different ratios of capture reagents attached to solid phases could be utilized in such an assay, to optimize analyte(s) detection.

In the embodiment described hereinabove, it is preferred that the specific binding member used as a capture reagent for the HIV-1 antibody analyte be HIV-1 p41 antigen , and that the specific binding member used as the capture reagent for the HIV-1 antigen analyte be anti-HIV-1 p24 antibody. It is most preferred that the HIV-1 p41 used be a recombinantly prepared antigen (protein). Also, it is preferred that the specific binding member for the antibody analyte indicator reagent is HIV-1 p41 antigen, labeled with an enzyme, and that the specific binding member for the antigen analyte indicator reagent is anti-HIV p24 antibody, labeled with an enzyme. It is most preferred this HIV-1 p41 antigen be recombinantly produced, and that the enzyme be horseradish peroxidase (HRPO).

In another embodiment of the present invention, a test sample suspected of containing any of the analytes of interest is simultaneously contacted with a first solid phase to which a first specific binding member of a first analyte and a first specific binding member of a second analyte have been attached, an indicator reagent for the first analyte comprising a specific binding member for the first analyte labeled with a signal generating compound and an indicator reagent for the second analyte comprising a specific binding member for the second analyte labeled with a signal generating compound, to form a mixture. The specific binding members serve as capture reagents

to bind the analyte(s) to the solid phases. If the specific binding member is an immunoreactant, it can be an antibody, antigen, or complex thereof, specific for each analyte of interest. If the specific binding member is an antibody, it can be a monoclonal or polyclonal antibody, an antibody fragment, a recombinant antibody, as well as a mixture thereof, or a mixture of an antibody and other specific binding members. The indicator reagents comprise specific binding members of the first and second analytes of interest which have been labeled with a signal generating compound. This mixture is incubated for a time and under conditions sufficient for a binding reaction to occur and which incubation results in the formation of capture reagent/first analyte/indicator reagent complexes of the first analyte and/or capture reagent/second analyte/indicator reagent complexes of the second analyte, if either or both the first or second analyte are present in the test sample. The presence of either analyte is determined by detecting the signal generated in connection with the complexes formed on either or both solid phases as an indication of the presence of the first analyte and/or the second analyte in the test sample. If the indicator employs an enzyme as the signal generating compound (label), then the signal can be detected visually or measured spectrophotometrically. Or, the label can be detected by the measurement of fluorescence, chemiluminescence, radioactive energy emission, etc., depending on the label used. Also, it is contemplated that the assay can include the use of a hapten-anti-hapten system, in which case the indicator reagent can further comprise a hapten such as biotin. The use of a biotin/anti-biotin system for assays is the subject matter of cop-pending U.S. Patent Application Serial No. 687,785 which corresponds to published European Patent Application No. 0160900 (published November 13, 1985), which enjoys common ownership and is incorporated herein by reference.

In the embodiment described hereinabove, it is preferred that the specific binding member used as a capture reagent for the HIV-1 antibody analyte be HIV-1 p41 antigen, and that the specific binding member used as the capture reagent for the HIV-1 antigen analyte be anti-HIV-1 p24 antibody. It is most preferred that the

Also, it is preferred that the specific binding member for the antibody analyte indicator reagent is HIV-1 p41 antigen, labeled with an enzyme. It is most preferred this HIV-1 p41 antigen be recombinantly produced, and that the enzyme be horseradish peroxidase (HRPO). Solid phases preferred include a magnetic or non-magnetic bead, a well of a reaction tray, and microparticles, either alone or in any combination.

Positive and negative controls can be included in the assay of the present invention to ensure reliable results. A blank solid phase(s), to which no capture reagent has been attached, can be utilized as the negative reagent control. Positive controls can include a positive control for each analyte which control is tested separately, and a combined positive control wherein the presence of all analytes to be detected in the assay are determined.

As previously stated, it is preferred that recombinantly-prepared antigens or synthetic peptides be used in the assay. However, it is to be understood that the present invention is not limited to the combination of recombinant proteins reagents and synthetic peptide reagents as described above, but that other combinations are contemplated. For example, one or more or all of the capture reagent proteins can be synthetically produced as can the protein of the lableld reagents. Moreover, viral lysates or isolates of the specific analyte can be employed, provided that the labeled HIV-2 synthetic peptide reagent of the present invention is employed. Expression of HIV gp41 or parts of HIV gp41 have demonstrated the utility of recombinant DNA (rDNA) derived HIV envelope sequences in diagnostic assays. Wood et al., Cold Spring Harbor Symposium on RNA Tumor Viruses, Cold Spring Harbor, New York, May 22-26 (1985); Chang et al., Biotechnology 3:905-909 (1985); Crowl et al., Cell 41:979-986 (1985); Cabradilla et al., Biotechnology 3:128-133 (1986). While it is general knowledge that viral proteins expressed in E. coli or other organisms have potential utility in diagnostic assays, development of immunoassays using these reagents, which also will have the specificity and sensitivity equal to or greater than the native viral proteins derived from the cell culture has been a difficult task.

Further, the expression of HIV gag proteins in E. coli have indicated that the HIV gag proteins produced by rDNA technology could have potential diagnostic value. Wood et al., Cold Spring Harbor Symposium on RNA Tumor Viruses, Cold Spring Harbor, New York, May 22-26 (1985); Dowbenko et al., PNAS USA 82:7748-7752 (1985); Ghrayeb et al., DNA 5:93099 (1986); Steimer et al., Virology 150:283-290 (1986).

The present invention utilizes recombinantly-produced HIV envelope proteins as assay reagents. The cloning of the HIV genome and expression of HIV envelope and core protein in E. coli, the purification and characterization of gp41 and p24, and various assay formats which utilize these recombinant proteins are described in U.S. Patent Application Serial No. 07/020,282 filed February 27, 1987 and previously incorporated herein by reference, from which this present invention claims priority. Briefly, HIV-infected HT-9 cells were harvested and total cellular DNA was isolated an subjected to digestion. The DNA segments encoding for the core protein and for the envelope glycoprotein were further subcloned into bacterial expression vectors using well-known recombinant technology. U.S.Patent Application Serial No. 07/020,282 also teaches that, in the detection of HIV-1 antibody, the use of recombinant antigens as the capture reagent and the indicator reagent allows for the detection of anti-HIV-1 antibodies of different immunoglobulin classes. These immunoglobulin classes include IgG, IgA, IgE and IgM. The detection of anti-HIV-1 IgG, IgM and IgA using the Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA assay has been described in an abstract by J. L. Gallarda et al., 5th Annual Forum on AIDS, Hepatitis and Other Blood-Borne Diseases, Atlanta, Georgia, March 29-April 1, 1992.

It is contemplated and within the scope of the present invention that recombinant antigens produced in heterologous sources can be utilized in the assay and will contribute an even greater lessening of false positive results. For example, if an E.coli prepared recombinant antigen such as p41 is used as the capture reagent, then a recombinant antigen p41 produced in any suitable source different than E. coli, such as in a suitable yeast host or other suitable host such as B. megaterium, can be used. The use

of heterologous sources of antigens in assays , including recombinant antigens, is the subject matter of co-pending U.S. Patent Application Serial No. 07/701,626, which enjoys common ownership and is incorporated herein by reference.

Further, although the present invention preferably utilizes recombinantly produced antigens, it is well within the scope of the invention to utilize synthetic proteins instead of recombinantly produced antigens. Thus, various synthetically prepared HIV peptides, of varying length, can be used.

The present invention also utilizes antibodies which specifically bind to HIV antigen analytes. In a preferred embodiment, anti-HIV p24 antibody is used. In a most preferred embodiment, a mixture of monoclonal antibodies, both specific for HIV p24 antigen, is used. In this mixture, one monoclonal antibody which specifically binds to an epitope on HIV-1 p24 to which epitope human anti-HIV-1 p24 IgG does not competitively bind is used with another monoclonal antibody which specifically binds to a different epitope of HIV-1 p24 to which different epitope human anti-HIV-1 p24 IgG does competitively bind. Further, the monoclonal antibody which does not competitively bind human anti-HIV-1 p24 IgG also specifically binds to HIV-2 p24 antigen. These monoclonal antibodies and their use in HIV antigen assays are the subject matter of co-pending U. S. Patent Application Serial No. 07/204,798, which enjoys common ownership and is incorporated herein by reference. These monoclonal antibodies are designated as 31-42-19 and 31-90-25. Hybridoma cell line 31-42-19 producing monoclonal antibody 31-42-19 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9726. Hybridoma cell line 31-90-25 producing monoclonal antibody 31-90-25 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9725. The use of these monoclonal antibodies as antibody fragments in HIV antigen assays also has been described in U.S. Patent No. 7,204,798, which enjoys common ownership and is incorporated herein by reference.

It is contemplated and within the scope of the invention that the detection of HIV-2 antigen is possible with the assay of the invention. In this assay format, HIV-2 p41 would be attached to a solid support as the HIV-2 antigen capture reagent, in addition to the previously-described HIV-1 p41 antigen capture reagent and HIV-1 antibody capture reagents. The solid support can be the same solid support to which all other capture reagents are attached, it can be the same solid support to which HIV-1 recombinant antigens have been attached, or it can be attached to a solid phase to which no other capture reagent (except for HIV-2 p41) has been attached. The assay procedure would be the same as described hereinabove for the various embodiments of the invention. The HIV-2 antibody analyte indicator reagent would comprise HIV-2 p41 antigen attached to a detectable label. In a preferred embodiment, it is preferred that recombinantly prepared HIV-2 p41 is utilized. The sequence for the HIV-2 virus (including p41 antigen) is described in EP 0 347,365, published December 20, 1989 to Diagen Corp, which is incorporated herein by reference. A most preferred HIV-2 recombinant antigen encodes the first 104 amino acids of the HIV-2 p41 antigen. The resulting plasmid designated as pJC104 expresses the HIV-2 env protein as a fusion with CKS protein. This plasmid encodes a recombinant protein containing the first 239 amino acids of the CKS protein, 13 amino acids from the pTB210N multiple restriction site linker, 104 amino acids from the HIV-2 env protein (amino acids 506-609), and an additional 15 amino acids from the pTB210N multiple restriction site linker, following the methods disclosed by Bolling and Mandecki, "CKS Method of Protein Synthesis," U.S. Patent Application Serial No. 167,067, filed March 11, 1988, which enjoys common ownership and is incorporated herein by reference.

The present invention will now be described by way of examples, which are meant to illustrate, but not to limit, the spirit and scope of the invention.

EXAMPLES

Example 1

Coating Procedure Using Two Solid Phases

This procedure utilized 1/4 inch polystyrene beads (available from Abbott Laboratories, Abbott Park, IL 60064) and a 60-well polystyrene reaction tray (available from Abbott Laboratories, Abbott Park, IL 60064). Two different anti-HIV-1 p24 monoclonal antibodies were coated on the beads, as follows. The beads were coated at a concentration of 8 μg/ml (approximately 1.6 μg/ml/bead) in a 0.25 M sodium citrate buffer (pH 7.2) for two hours at 45°C. The beads then were washed in the 0.25 M sodium citrate buffer (pH 7.2), and then they were reacted with a detergent solution containing 0.1% Triton X-100® (polyoxyethylene ether, available from Sigma Chemical Co., St. Louis, MO) for one hour at 45°C. The beads next were blocked with 1% bovine serum albumin (BSA) in 0.25 M sodium citrate buffer (pH 7.2) for 30 minutes at 45°C, and then overcoated with 2% sucrose, 1% phosphate glass for 15 minutes at 15-30°C in 0.25 M sodium citrate buffer and allowed to dry. The two monoclonal antibodies used are designated as 31-42-19 and 31-90-25. They are the subject matter of a patent application U.S. Patent Application Serial No. 07/204,798 that describes their development and uses, previously incorporated herein by reference. Their use also has been described in U.S. Patent No. 7,204,798, which enjoys common ownership and is incorporated herein by reference. Hybridoma cell line 31-42-19 producing monoclonal antibody 31-42-19 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9726. Hybridoma cell line 31-90-25 producing monoclonal antibody 31-90-25 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9725.

Next, the wells of the 60-well reaction tray were coated with HIV antigen, as follows. The recombinant protein HIV-1 p41 env protein designated as pTB319 was added to each well at a

concentration of 1 μg/ml in 0.1 M 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS buffer, pH 11), and incubated for two hours at 40°C. The wells then were washed twice with 400 μl of phosphate buffered saline (PBS, pH 7.5), reacted with 0.1% Tween-20® for one hour at 40°C, and then blocked with 3% BSA in PBS for one hour at 40°C. The wells next were overcoated with 5% sucrose in PBS for 20 minutes at room temperature and allowed to dry.

The pTB319 plasmid producing recombinant protein pTB319 is the subject matter of a patent application to Bolling and Mandecki, "CKS Method of Protein Synthesis," U.S.Patent Application Serial No. 167,067, filed March 11, 1988, previously incorporated herein by reference. pTB319 was produced by inserting a synthetically-produced DNA fragment which encoded the carboxy terminal 42 amino acids of HIV-1 p120 into the plasmid pTB315, as described in Bolling and Mandecki, Ibid.

Example 2

Simultaneous Assay for HIV antigen and HIV antibody

The two solid phases prepared as described in Example 1 were used in an assay for detection of HIV antigen and/or HIV antibody in a test sample, as follows. An HIV-1 seroconversion panel, which contained 65 serum samples derived from nine HIV-1 infected individuals undergoing seroconversion, was used in the assay. Each serum sample was diluted in a separate well of the 60-well tray previously prepared in Example 1 by adding 150 μl of the serum sample to 50 μl of specimen diluent, which contained 2% Tween 20® (polyoxyethylenesorbitan, available from Sigma Chemical Co., St. Louis, MO). Then, a bead previously coated with anti-HIV p24 antibodies as described in Example 1 was placed in each well containing a serum sample. The wells of each tray were incubated for 60 minutes at 40°C under continuous rotation. Following incubation, each well of the 60-well reaction tray was washed with 15 ml of deionized water (dH_2O) in the Abbott Parallel Processing Center™ (PPC, available from Abbott Laboratories, Abbott Park, IL). 200 μl of an HIV p24 antibody probe reagent (rabbit polyclonal $F[ab']_2$ anti-HIV-1 [active ingredient: anti-p24 antibody at a concentration of 2 to 6 μg/ml] in an antibody diluent

(2.25% BSA, 7.5% calf serum, 7.5% goat serum, 25% human recalcified plasma, 0.1% sodium azide) was added to each well/bead and then the resulting mixture was incubated for 60 minutes at 40°C without rotation. Each bead/well in the reaction tray was washed with 15 ml of dH_2O. Then, 200 µl of conjugate diluent (0.18% Tris, 1.19 % Tris-HCl, 0.38% NaCl, 9.0% calf serum, 0.9% goat serum, 10.0% human calcified plasma, 4.5% Triton X-100®, 0.013% gentamicin sulfate, 0.009% thimerosal) which contained a mixture of recombinant HIV-1 p41 antigen labelled with horseradish peroxidase (pTB319 coupled to HRPO), and HRPO-labelled goat anti-rabbit IgG antibody were added to each bead/well of the reaction tray and allowed to incubate for 60 minutes at 40°C without rotation. Each bead/well of the 60-well reaction tray was washed with 15 ml of dH_2O as previously described. Then, 300 µl of o-phenylenediamine-2HCl (OPD) was added to each well/bead and then was incubated for 30 minutes at room temperature in the dark. The reaction then was stopped by adding 300 µl of a stopping reagent (1 N H_2SO_4) to each well/bead. The reaction was read using the Abbott PPC which measured the optical density of the reaction at 492 nm using a 630 nm reference. The cutoff value was established as 0.1 OD + mean OD of the negative control. Thus, serum samples were considered reactive (positive) if the sample to cutoff value was greater than 1.

All 65 serum samples from the 9 individuals described hereinabove were tested following this procedure. The results obtained then were compared to the results obtained for the same serum sample when using an HIV antigen assay (HIVAG®, available from Abbott Laboratories, Abbott Park, IL) and an HIV antibody assay (Human Immunodeficiency Virus Types 1 and 2: E. coli and B. megaterium, recombinant antigen, Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA; available from Abbott Laboratories, Abbott Park, IL) following manufacturer's directions as provided in each product insert. The data are reported in Table 1, wherein "OD" refers to the optical density reading, "S/CO" means sample/cut-off value, "Result." refers to the interpretation of the test, "HIV-1/2 Ab HIV-1 Ag Comb" designates the assay of the invention, "HIV-1/2 Ab" designates the HIVAB® HIV-1/HIV-2

(rDNA) EIA assay and "HIV-1 Ag" designates the Abbott HIVAG® assay.

TABLE 1

Sample ID		HIV-1/2 Ab		HIV-1/2 Ab HIV-1 Ag Comb		HIV-1 Ag	
		S/CO	Result	S/CO	Result	S/CO	Result
SV0021	1	1.34	+	0.33	-	1.10	+
	2	1.92	+	2.55	+	1.80	+
	3	3.15	+	6.32	+	2.20	+
	4	1.79	+	3.65	+	0.60	-
SV0031	5	0.83	-	0.13	-	0.40	-
	6	0.87	-	0.13	-	0.30	-
	7	0.86	-	0.13	-	0.35	-
	8	0.78	-	0.15	-	0.35	-
	9	0.95	-	0.17	-	0.40	-
	10	0.80	-	0.13	-	0.38	-
	11	0.62	-	0.10	-	0.35	-
	12	3.40	+	9.08	+	13.20	+
	13	3.40	+	10.09	+	8.50	+

	14	3.40	+	3.00	+	10.50	+
	15	3.40	+	3.28	+	6.40	+
	16	1.87	+	5.25	+	3.20	+
	17	1.52	+	7.34	+	0.80	-
	18	1.38	+	7.28	+	1.20	+
SV0051	19	0.97	-	0.40	-	1.70	+
	20	2.21	+	1.49	+	15.00	+
	21	2.55	+	2.66	+	7.90	+
	22	2.54	+	7.30	+	2.20	+
	23	2.57	+	5.51	+	1.80	+
	24	1.89	+	3.81	+	0.40	-

TABLE 1 (cont.)

Sample ID		HIV-1/2 Ab		HIV-1/2 Ab HIV-1 Ag Comb		HIV-1 Ag	
		S/CO	Result	S/CO	Result	S/CO	Result
SV0091	25	2.40	+	0.24	-	18.80	+
	26	3.40	+	0.44	-	71.60	+
	27	3.40	+	2.81	+	5.20	+

	+	28	3.40	+	2.75	+	1.90
	-	29	2.71	+	2.43	+	0.65
	-	30	2.23	+	2.25	+	0.65
	-	31	1.54	+	3.72	+	0.49
	-	32	1.35	+	10.57	+	0.41
		33	nt*				
SV0111	+	34	3.40	+	0.12	-	32.80
	+	35	3.40	+	0.45	-	42.90
	+	36	3.40	+	11.23	+	20.70
	+	37	2.35	+	4.16	+	1.40
	+	38	1.82	+	3.81	+	1.10
	-	39	1.71	+	3.34	+	0.80
	-	40	1.40	+	5.49	+	0.72
SV0161	+	41	1.01	+	0.12	-	1.44
	+	42	1.21	+	1.14	-	3.82
	+	43	2.17	+	0.79	-	8.41
	+	44	3.40	+	10.25	+	11.80

SV0061	+	47	1.13	+	0.20		2.90
	+	48	1.23	+	0.26	-	3.60
	+	49	3.33	+	1.93	+	10.90
	+	50	3.09	+	1.72	+	13.50
	+	51	1.93	+	0.96	-	7.10
	+	52	1.88	+	1.39	+	4.40
	+	53	1.30	+	1.60	+	1.90
	-	54	1.45	+	3.10	+	0.97
	-	55	1.22	+	4.28	+	0.98
SV0071	-	56	0.97	-	0.13	-	0.40
	-	57	0.84	-	0.14	-	0.40
	+	58	3.19	+	2.97	+	2.30
	-	59	1.82	+	2.37	+	0.52
	-	60	0.97	-	2.84	+	0.42

TABLE 1 (cont.)

Sample ID	HIV-1/2 Ab HIV-1 Ag Comb S/CO	Result	HIV-1/2 Ab S/CO	Result	HIV-1 Ag S/CO	Result
SV0081 61	0.80	-	0.12	-	0.45	-
62	0.85	-	0.15	-	0.32	-
63	0.83	-	0.14	-	0.49	-
64	1.41	+	3.40	+	0.43	-
65	1.10	+	4.96	+	0.37	-
66	1.23	+	8.58	+	0.37	-
No. Positive/No. Tested	51/65		41/65		35/65	

*nt: This sample of the seroconversion panel was unavailable for testing.

The data from Table 1 indicates that the method of the present invention had greater sensitivity than either the HIV-1/2 Ab or the HIV-1 Ag test when the results from the three individual tests were compared separately to each other. It is expected that the assay can be optimized even further to detect both HIV p24 antigen which is present early in the course of infection and also in the final stages of HIV infections, as well as HIV antibodies which appear later in infection at the time of seroconversion.

Example 3

Coating Procedure For One Solid Phase

In this procedure, only one solid phase was coated with HIV antigen and HIV antibody, as follows. Into each well of a 96-well microtiter plate (Immulon 4®, available from Dynatech, Alexandria VA) monoclonal antibody 31-42-19, monoclonal antibody 31-90-25 and recombinant HIV-1 p41 antigen designated as pTB 319 (as described in Example 1) were coated at a concentration of 1 μg/ml each in 0.1 M carbonate buffer (pH 9.5) for two hours at room temperature. The wells next were blocked with 300 μl of blocking reagent (comprising 5% non-fat dry milk, 10 mM Tris [pH 8.0] 150 mM NaCl and 0.05% Tween-20®) for one hour at room temperature.

Example 4

HIV Antigen/Antibody Assay Using One Solid Phase

The solid phase prepared as described in Example 3 was used in an assay to detect the presence of HIV antigen and/or HIV antibody in a test sample, as follows. Each serum sample of a 12-member seroconversion panel (Panel G available from Boston Biomedica Inc., Boston MA) as well as positive and negative controls were tested. 150 μl of each serum sample or positive or negative control was diluted in a separate well of the microtiter plate with 50 μl of specimen diluent (containing 15 μl of Triton X-100® and 35 μl of blocking reagent, as described in Example 3) and incubated for 60 minutes at room temperature without rotation. After incubation the wells were washed with eight cycles of 300 μl of washing buffer (0.05% non-fat dry milk, 10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20®) using a Nunc 8-channel "Immunowash" manifold (available from Nunc, Denmark). Next, 175 μl of of an HIV p24 antibody probe reagent (rabbit polyclonal $F[ab']_2$ anti-HIV-1 [active ingredient: anti-p24 antibody at a concentration of 2 to 6 μg/ml) in an antibody diluent (2.25% BSA, 7.5% calf serum, 7.5% goat serum, 25% human recalcified plasma, 0.1% sodium azide) were added to each well and incubated for 60 minutes at room temperature without rotation. After incubation the wells were washed with eight cycles of wash buffer as previosuly described. Then, 150 μl of conjugate diluent (as

described in Example 2) which contained a mixture of recombinant HIV-1 p41 protein (pTB 319) labelled with HRPO and HRPO-labelled goat anti-rabbit IgG (previously described in Example 2) were added to each well and incubated for 60 minutes at room temperature without rotation. The wells were washed with eight cycles of wash buffer (described previously herein) and then rinsed with dH_2O. Then, 125 µl of OPD substrate was added to each well and the wells were incubated at room temperature for 10 minutes in the dark. The reaction was stopped by adding 125 µl of stopping reagent (previously described in Example 2). The absorbance of each well was read at 490 nm with a 630 nm reference. The cutoff value of .025 OD + mean OD of the negative control was established. Samples were considered reactive (positive) when the sample/cutoff value was greater than 1

The data from these assays are presented in Table 2. In Table 2, "OD" refers to the optical density reading, "S/CO" means sample/cut-off value, "Result." refers to the interpretation of the test, "NC" refers to negative control and "PC" refers to the positive control.

TABLE 2

Sample ID	HIV-1/2 Ab HIV-1 Ag Comb S/CO	Result	HIV-1/2 Ab S/CO	Result	HIV-1 Ag pg/ml*	Result
NC	0.85	-				
NC	0.72	-				
NC	0.82	-				
PC	1.35	+				
PC	1.13	+				
1	1.22	+	0.09	-	>200	+
2	1.64	+	0.23	-	>200	+
3	2.33	+	2.55	+	>200	+
4	4.04	+	4.07	+	155	+
5	2.42	+	1.92	+	40	+
6	1.03	+	1.95	+	5	+
7	1.09	+	4.87	+	0	-
8	1.57	+	7.31	+	0	-
9	1.61	+	7.31	+	0	-
10	1.79	+	9.40	+	0	-

11	3.76	+	>17.85	+	0
-					
12	5.62	+	>17.85	+	0
-					
TOTALS	12/12		10/12		6/12

*pg/ml - picograms/ml

As the data from Table 2 demonstrate, the assay of the invention was capable of detecting the presence of HIV antibody and/or HIV antigen in the seroconversion panel. Compared individually to the HIV-1/2 Antibody test, and HIV-1 Antigen test, the method of the present invention was more sensitive at detection than either test alone, based on detection of either antigen or antibody. When the results from Table 2 of the method of the present invention are compared to the combined results of the HIV-1/2 antibody test and the HIV antigen test, the method of the present invention was able to detect all specimens that were reactive by either test.

Example 5

Coating Microparticles Simultaneously With HIV Antibody and HIV Antigen Capture Reagents

Both monoclonal anti-HIV p24 antibodies previously described (31-42-19 and 31-90-25) and the recombinant HIV-1 p41 antigens previously described (HIV-1 p41 recombinant protein pTB319, and HIV-2 p41 recombinant protein pJC104) are together and simultaneously coated onto a uniform 0.5% suspension (wt/volume) of polystyrene microparticles (available from Seradyne Inc., Indianapolis, Indiana) at concentrations of 150 μg/ml each in 0.01 M carbonate buffer (pH 9.5) for two hours at room temperature (15-30°C). The suspension of microparticles is briefly centrifuged and the microparticle pellet is resuspended in 0.05M Tris buffer (pH 8.0) to wash away excess uncoupled protein. This washing is repeated until no uncoupled protein remains. After blocking the microparticles with 10 mg/ml casein in 0.01 M Tris (pH 8.0), 0.15 M NaCl at 56°C for 18-24 hours, the microparticles again are

Simultaneous Detection of HIV Antibody and HIV Antigen Using Microparticles

The Abbott IM_x® Microparticle Enzyme Immunoassay (MEIA) system is used, although any system which employs microparticles can be used. The Abbott IM_x® MEIA system is thoroughly described in the Abbott IM_x® Operation and Customer Training Manuals (available from Abbott Diagnostic Division, Abbott Laboratories, Abbott Park, IL). In this assay, 100 µl of the 0.15% suspension prepared in Example 5 is mixed together with 100 µl of test sample suspected of containing HIV-1 and/or HIV-2 antibody and/or HIV-1 antigen, and incubated at 40°C for ten minutes in an Abbott IM_x® reaction cell to form a reaction mixture. HIV antibodies and/or HIV antigens bind to the microparticles in an antibody/antigen/microparticle complex. 150 µl of the reaction mixture is transferred onto a glass fiber matrix to which the microparticles are retained in an irreversible binding. The antibody/antigen/microparticle complexes then are reacted with 50µl of a probe consisting of biotinylated recombinant HIV-1 and HIV-2 recombinant p41 antigens (pTB319 and pJC104, previously described) and biotinylated $F(Ab')_2$ anti-HIV-1 p24 in 0.05M Tris 9pH8.0), 2% BSA, 0.25% saponin and 0.1% sodium azide at 40°C for ten minutes. 50 µl of an antibody conjugate consisting of goat anti-biotin alkaline phosphatase in 0.1 M Tris (pH 8.0), 0.5M NaCl, 0.9% Brij-35®, 1.0% BSA and 0.1% sodium azide then is allowed to react with the biotin probe/antibody/antigen/microparticle complexes for ten minutes at 40°C. Then, these microparticle complexes are washed six times with 0.05 M Tris (pH 8.0), 0.3 M NaCl and 0.1 % sodium azide, the biotin probe/antibody/antigen microparticle complex is reacted with 50 µl of the substrate methylumbelliferyl phosphase (MUP, Abbott Laboratories, Abbott Park, IL), and the fluorescence of the product , methylumbelliferon, (MU) is measured. The rate of MU

production is proportional to the concentration of analyte(s) in the test sample.

Example 7

Coating Microparticles Separately with HIV Antibody and HIV Antigen Capture Reagents

In this example, the various analyte capture reagents are coated separately onto polystyrene microparticles (available from Seradyne Inc., Indianapolis, Indiana). Each of the reagents may be coated separately from each other or in various combinations with each other. After each of the analyte capture reagents is coated on their respective microparticles, the various coated microparticles are pooled together and used in the assay.

In the present example, the two monoclonal anti-HIV p24 antibodies (31-42-19 and 31-90-25) are coated together onto microparticles separate from the microparticles coated simultaneously with recombinant HIV-1 and HIV-2 p41 antigens (pTB319 and pJC104). Although the exact amount may vary, in general, the coating procedure will approximate that described in Example 5. After blocking the physically separated microparticles with 10 mg/ml casein in 0.01 M Tris (pH 8.0), 0.15 M NaCl at 56°C for 18-24 hours, the microparticles again are washed as described in Example 5, pooled together, and diluted to a 0.015% suspension (wt/volume) in 0.05 M Tris 9pH 8.0), 0.15 M NaCl, 1% BSA and 15% sucrose. At the pooling step, the microparticles may be pooled at various ratios to affect sensitivity and specificity of the assay in order to optimize their use.

Example 8

Simultaneous Detection of HIV Antibody and HIV Antigen on Microparticles Separately Coated With HIV Antibody and HIV Antigen Capture Reagents

The Abbott IM_X® Microparticle Enzyme Immunoassay (MEIA) system is used, although any system which employs microparticles can be used. The Abbott IM_X® MEIA system is thoroughly described in the Abbott IM_X® Operation and Customer Training Manuals (available from Abbott Diagnostic Division,

Abbott Laboratories, Abbott Park, IL). In this assay, 100 μl of the 0.15% suspension prepared in Example 5 is mixed together with 100 μl of test sample suspected of containing HIV-1 and/or HIV-2 antibody and/or HIV-1 antigen, and incubated at 40°C for ten minutes in an Abbott IM_x® reaction cell to form a reaction mixture. HIV antibodies and/or HIV antigens bind to the microparticles in an antibody/antigen/microparticle complex. 150 μl of the reaction mixture is transferred onto a glass fiber matrix to which the microparticles are retained in an irreversible binding. The antibody/antigen/microparticle complexes then are reacted with 50μl of a probe consisting of biotinylated recombinant HIV-1 and HIV-2 recombinant p41 antigens (pTB319 and pJC104, previously described) and biotinylated $F(Ab')_2$ anti-HIV-1 p24 in 0.05M Tris 9pH8.0), 2% BSA, 0.25% saponin and 0.1% sodium azide at 40°C for ten minutes. 50 μl of an antibody conjugate consisting of goat anti-biotin alkaline phosphatase in 0.1 M Tris (pH 8.0), 0.5M NaCl, 0.9% Brij-35®, 1.0% BSA and 0.1% sodium azide then is allowed to react with the biotin probe/antibody/antigen/microparticle complexes for ten minutes at 40°C. Then, these microparticle complexes are washed six times with 0.05 M Tris (pH 8.0), 0.3 M NaCl and 0.1 % sodium azide, the biotin probe/antibody/antigen microparticle complex is reacted with 50 μl of the substrate methylumbelliferyl phosphase (MUP, Abbott Laboratories, Abbott Park, IL), and the fluorescence of the product , methylumbelliferon, (MU) is measured. The rate of MU production is proportional to the concentration of analyte(s) in the test sample.

Example 9

<u>Preparation and Expression of Recombinant HIV-1 Antigens</u>

<u>Preparation of initial HIV-1 and HIV-2 clones</u>

An HIV-1 genomic library was prepared by ligating a partial <u>Eco</u>RI digestion of genomic DNA derived from HIV-1 infected HT-9 cells (obtained from Dr. Robert Gallo, National Cancer Institute, Laboratories of Tumor Cell Biology, Lot No. P3-21) with bacteriophage lambda Charon 4A EcoRI arms and transfecting into <u>E. coli</u> C600. The library was screened by

hybridization with cDNA made from HIV-1 viral RNA, and a single phage (designated Phage 4B) was obtained containing the entire HIV-1 genome.

Phage 4B DNA was digested with KpnI and ligated into the KpnI site of pUC18 (Bethesda Research Laboratories). A clone (designated pcK2) containing the entire p41 region of the HIV-1 env gene was identified and mapped.

Phage 4B DNA was digested with EcoRI and ligated into the EcoRI site of pBR322. A clone (designated pcR23) containing the entire HIV-1 gag gene was identified and mapped.

A DNA fragment containing the/env gene from HIV-2 prophage isolate D1945 was identified within a lambda genomic library of prophage DNA. This fragment was subcloned into an EcoRI site of an E. coli expression vector (lambda PL vector pKH20). The resulting plasmid was named pEHa.

Preparation of recombinant HIV-1 gp41 antigen.

The construction of the envelope expression vector was a two step process. The first step involved the construction of an E. coli plasmid containing a smaller DNA fragment containing env (designated p41C). The second step involved the construction of an expression vector with the ability to survive in both Escherichia Sp. and Bacillus sp., and the introduction of the env fragment into this plasmid (designated pOM10).

An 854 base pair (bp) BglII/BamHI DNA fragment obtained from plasmid pcK2 was ligated into the BamHI site of pUC9 (Pharmacia). A clone containing a part of the env gene in the same orientation as the lacZ gene was identified, mapped, and designated p41A. A 557 base pair bp BamHI DNA fragment obtained from plasmid pcK2 was ligated into the BamHI site of plasmid p41A. A plasmid containing the complete p41 sequence of the env gene in the same orientation as the lacZ gene was identified, mapped, and designated p41C.

An E. coli plasmid containing the Bacillus sporulation promoter spoVG (developed by Dr. R. Losick, Harvard University, and designated pVG1) was restricted with SmaI. This DNA fragment was ligated into the Bacillus plasmid pE194 which had

previously been restricted with XbaI and blunt ends were formed using E. coli DNA polymerase 1 (Klenow fragment) to fill in the "sticky" DNA ends (blunt-end treatment). A plasmid (designated pAS5) was isolated, mapped and shown to have the ability to survive in both E. coli and B. subtilis. The env gene was then inserted into pAS5. A DNA fragment from the plasmid p41C containing the env gene was generated via EcoRI/SalI digestion and subsequent blunt-end treatment. This DNA fragment was ligated to plasmid pAS5 which had been linearized with SalI and blunt-end treated. One isolated clone (designated pAS14) was determined to have the env gene fused to the spoVG promoter in the proper orientation.

Finally, the erythromycin resistance gene in pAS14 was replaced by the chloramphenicol resistance gene from a related Bacillus plasmid pC194 as follows. A 1107 bp DNA fragment containing the chloramphenicol acetyl transferase (CAT) gene from a ClaI/DraI digest of the plasmid pC194 was isolated. This DNA fragment was ligated to the 6407 bp DNA fragment isolated from a ClaI/SmaI digestion of pAS14 (a treatment which removes all of the original erythromycin resistance gene). The final plasmid obtained was designated pOM10.

The promoter region, transcriptional start, and ribosomal binding site span bases 4840-4971. The coding region (bases 4972-6183) consists of sequences derived from the spoVG region of the parent plasmid pVG1 (bases 4972-5004), sequences derived during DNA ligations (bases 5005-5010) and sequences derived from the HIV-1 env gene gp120 (bases 5011-5145) (Ratner, L., et. al. Nature 313:277-284, 1985). The p41 sequences are from bases 5146-6180. The translation is terminated at the native termination codon of the env gene (bases 6181-6183). The DNA sequence coding for the recombinant protein was confirmed by sequencing of the plasmid harvested after fermentation for three lots. The sequenced lots were fermented from the same cell bank used to produce antigen for clinical master lots.

Plasmid pOM10 expresses the HIV-1 envelope protein as a fusion protein containing 11 amino acids derived from the amino-terminus of the spoVG protein, 2 amino acids derived as a result of

DNA manipulations during ligations, followed by the final 45 amino acids from the pl20 envelope protein and the entire p41 protein sequence. This protein is referred to as recombinant p41 (rp41).

Preparation of recombinant HIV-1 gp41 fusion protein

The construction of this recombinant E. coli clone expressing the HIV-1 CKS-120141 fusion antigen was carried out in several steps. First the gene for the gp41 portion of the HIV-1 antigen was synthesized and inserted into a modified pUC18 giving the plasmid pTB315. Next, the DNA sequence coding for the carboxyl 42 amino acids of the gpl20 protein was synthesized and inserted into pTB315 resulting in plasmid pTB316. Finally, the gpl20/41 gene was transferred to an expression plasmid (pTB210) which allowed efficient expression of the antigen as a fusion protein. The resulting plasmid, pTB319, was isolated and mapped.

A gene encoding the amino acids 519-673, and 712-863 of the HIV-1 gpl60 envelope protein (Ratner et. al., Nature 313:277-284, 1985) was designed to be constructed from a series of synthetic DNA fragments in a pUC18 plasmid derivative.

Fourteen fragments were chemically synthesized, reproducing a portion of the published gp41 sequence. This sequence consists of amino acids 519-673 and 712-863 with a 38 amino acid transmembrane region from amino acids 674-711 deleted. The 14 synthetic fragments were subcloned into pWM500 (Mandecki and Bolling, Gene 68:101-107, 1988), purified and ligated together to form the gp41 portion of the fusion protein. However, at amino acids 741 and 742, an A/T deletion occurred resulting in a 14 amino acid frameshift and premature translation termination in fragment 9. The resultant synthetic DNA sequence retains flanking BamHI and KpnI sites for insertion into a modified pUC18 plasmid with its NarI site destroyed by the insertion of a linker, designated pMB10.5.

A 129 base pair double stranded DNA fragment representing the carboxy-terminus of gpl20 was synthesized (311.3 and 311.4) and inserted into the remaining NarI site of pTB315. This fragment was inserted into plasmid pTB315 which was digested

screened such that the orientation of the inserted fragment was in the same orientation as the gp41 gene.

This plasmid, derived from plasmid pBR322, contains a modified lac promoter fused to a kdsB gene fragment (encoding the first 239 of the entire 248 amino acids of the E. coli CMP-KD0 Synthetase or CKS protein), and a synthetic linker fused to the end of the kdsB gene fragment. The synthetic linker includes multiple restriction sites for insertion of genes, translational stop signals and the trPA rho-independent transcriptional terminator. This plasmid encodes 239 amino acids of CKS and 22 amino acids coded for by the synthetic linker.

Plasmid pTB316 was digested with BamHI and KpnI and a 1073 bp fragment was isolated. This fragment consisted of the original synthetic gp41 gene with the carboxyl 42 amino acids of the gpl20 gene inserted in the proper location. This fragment was inserted into pTB210 which was previously digested with BglII and KpnI. The resulting plasmid, designated pTB319, was isolated and mapped.

The promoter region, transcriptional start, and ribosomal binding site span bases 45-125. The coding region is comprised of sequences derived from the 239 amino acids of the CKS protein (bases 126-842) and the 11 amino acids from the synthetic polylinker (bases 843-875). This is followed by 42 residues of the pl20 HIV-1 env (bases 876-1001) and 185 residues of the HIV-1 p41 env (bases 1002-1556). The 38 amino acid deletion of the transmembrane region is between base pairs 1466 and 1467. Finally, there are an additional 14 amino acids (bases 1557-1598) as the result of a frameshift due to a single A/T deletion and a premature translational termination (bases 1599-1601). The DNA sequence coding for the recombinant protein was confirmed by sequencing of the plasmid harvested after fermentation for three lots.

The plasmid pTB319 encodes a recombinant protein containing 239 amino acids of the CKS protein and 11 amino acids from the pTB210 multiple restriction site linker. This is followed by 42 amino acids from the carboxyl end of HIV-1 pl20, 185 amino

acids from the HIV-1 p41 protein (a truncated protein with a 38 amino acid deletion of amino acids 674-711 (Ratner et. al., Nature Vol 313:277-284, 1985) spanning the p41 transmembrane region). Finally there are 14 amino acids resulting from a frameshift and premature termination due to a single A/T deletion between nucleotides 1556 and 1157. This protein is referred to as recombinant pCKS-41 (rpCKS-41).

Preparation of recombinant HIV-1 p24 antigen.

The construction of the p24 gag expression vector was a multi-step process. The first step involved the construction of an E. coli plasmid, pB1, with a smaller gag containing DNA fragment. The second step involved the construction of an expression vector, designated pKRR951, with the proper molecular signals to allow efficient expression. Finally, molecular information was added to the plasmid to allow regulation of gene expression resulting in the final plasmid pKRR955.

A 949 bp PvuII/BglII DNA fragment obtained from plasmid pcR23 was ligated into the plasmid pUC9 (Pharmacia) previously digested with HindII and BamHI. A clone containing a part of the gag gene (including the p24 coding region) in the same orientation as the lacZ gene was identified, mapped, and designated pB1.

The gag gene DNA fragment was then introduced into an expression vector pKRR810 which placed the gag gene expression under the control of the E. coli lambda phage P_L promoter while allowing efficient termination of protein synthesis. A 963 bp DNA fragment containing most of the gag gene was obtained by an EcoRI (complete)/PstI (partial) digestion of plasmid pB1. A synthetic oligonucleotide DNA fragment of 36 bp was added to the gag gene fragment to reconstruct the amino-terminus of the encoded protein and to place an EcoRI site immediately upstream of the initiation codon. This modified fragment was inserted into the EcoRI site of the expression vector pKRR810. A clone (pKRR950) with the gag gene in the same orientation as the phage P_L promoter was identified, isolated and mapped. The size of this clone was reduced by 106 bp by ApaI digestion and religation of the pKRR950 plasmid resulting in a plasmid designated pKRR951.

lambda cI_{ts} regulatory gene and the E. coli lambda phage P_R promoter were included within the construct. The addition of this temperature sensitive gene allows control of the lambda promoters and subsequently of the gag gene expression. A 2392 bp DNA fragment containing the lambda cI_{ts} regulatory gene and the E. coli lambda phage P_R promoter was obtained via BglII digestion of a plasmid called pRK248.cI_{ts}. This fragment was inserted into the BglII site of plasmid pKRR951 resulting in plasmid pKRR955.

The promoter region, transcriptional start, and ribosomal binding site span bases 7757-271. This region is derived from two different lambda phage mutants and a synthetic region. The coding region is comprised of a synthetic sequence which duplicates the NH_2 end of the lacZ' gene from pUC9 (bases 272-307), sequences coding for a portion of the HIV-1 gag gene (bases 308-1183) including the entire p24 sequence (bases 344-1036), followed by a short sequence from the synthetic three frame translation terminator of the vector pKRR810 (bases 1169-1180). Translation is terminated at the third termination codon in this segment (bases 1181-1183). The sequence shows the $rrnBt_1$ transcription terminator (bases 1184-1241).

The plasmid pKRR955 produces a fusion protein comprised of 12 amino acids derived from the lacZ protein and the pUC9 polylinker region. followed by a portion of the gag protein (including the final 12 amino acids of the pl7 protein, the entire 231 amino acids of the p24 protein and the first 44 amino acids of the pl5 protein). followed by 4 amino acids derived from the terminator portion of the pKRR810 vector. This protein is referred to as recombinant p24 (rp24).

Preparation of recombinant HIV-2 gp36 env antigen.

The construction of this recombinant E. coli clone expressing the rp41 HIV-2 antigen was carried out in two steps. First a fragment of the HIV-2 env gene was isolated from a HIV-2 prophage and subcloned into an E. coli expression vector designated pEHa. Second, a HIV-2 env gene fragment was

subcloned from plasmid pEHa into an alternative expression vector, pTB210N, resulting in the plasmid pJC104.

A DNA fragment containing the env gene from HIV-2 (prophage isolate D194.5) was identified within a lambda genomic library of prophage DNA. This fragment was subcloned into an EcoRI site of an E. coli expression vector (lambda P_L vector pKH20). The resulting plasmid was named pEHa. This work was done by DIAGEN GmbH, Neiderheider Strasse 3, 4000 Dusseldorf (Kuhnel et. al. Proc. Natl. Acad. Sci. USA 86:2383-2387, 1989).

The cloning vector pTB210 allows the fusion of recombinant genes to the CKS protein. This plasmid consists of the plasmid pBR322 with a modified lac promoter fused to a kdsB gene fragment (encoding the first 239 of the entire 248 amino acids of the E. coli CMP-KD0 Synthetase or CKS protein), and a synthetic linker fused to the end of the kdsB gene fragment. The synthetic linker includes: multiple restriction sites for insertion of genes, translational stop signals, and the trpA rho-independent transcriptional terminator.

The plasmid pTB210N contains a NcoI site in the synthetic linker and is derived from the plasmid pTB210.

Plasmid pEHa was digested with NcoI and a 314 base pair fragment encoding the first 104 amino acids of the HIV-2 p41 protein was isolated and inserted into the NcoI site of plasmid pTB210N. This plasmid, designated pJC104, expresses the HIV-2 env protein as a fusion with the CKS protein.

The promoter region, transcriptional start, and ribosomal binding site span bases 45-125. The coding region is comprised of sequences derived from the 239 amino acids of the CKS protein (bases 126-842) and the 13 amino acids from the synthetic polylinker (bases 843-881). This is followed by 104 residues of the amino end of the HIV-2 env (bases 882-1193) and 15 amino acids of the remainder of the polylinker (bases 1194-1238). The translation is terminated at the termination codon at bases 1239-1241.

The plasmid pJC104 encodes a recombinant protein containing the first 239 amino acids of the CKS protein, 13 amino acids from the pTB210N multiple restriction site linker, 104 amino acids from the HIV-2 env protein (amino acids 506-609 of the HIV-2

env protein), and an addi... multiple restriction site linker. This protein is referred to as recombinant p41 HIV-2 (rp41 HIV-2.

Plasmid host cell systems

The plasmid pOM10, prepared as described above, was transformed into protoplasts of B. megaterium strain PY361 (a prototrophic derivative of strain QMB1551 cured of native plasmids) and viable chloramphenicol resistant cells were allowed to regenerate. Expression of rp41 antigen was under the control of the spoVG promoter and was observed when the cells entered the sporulation growth phase. This plasmid replicated as an independent element, was non-mobilizable, and was maintained at approximately 10 to 30 copies per cell.

The plasmid pTB319 prepared as described above, was transformed into E. coli K-12 strain XL-1 (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac-/F', proAB, lacI^{q}ZdeltaM15, TN10) cells made competent by the calcium chloride method. In this construction the expression of the rpCKS-41 protein is under the control of the lac promoter. Recombinant pCKS-41 expression was induced by the addition of IPTG to 100μg/ml. This plasmid replicated as an independent element, was non-mobilizable and was maintained at approximately 10 to 30 copies per cell.

The plasmid pKRR955 prepared as described above, was transformed into E. coli K-12 strain KRR136 (Dlac-pro, supE, thi-1, rpsL, sbcB15, endA, hsdR4, lon-9, tsx:-462:TnlO/F', traD36, proAB+ lacI^{q}ZdeltaM15) cells made competent by the calcium chloride method. In this construction the expression of rp24 protein was under the control of both the lambdaP$_L$ and lambdaP$_R$ promoters and the cI$_{ts}$ repressor expressed from the cI$_{ts}$ gene present on the plasmid. Recombinant p24 expression was induced by temperature shift from 30°C to 42°C. This plasmid replicated as an independent element, was non-mobilizable and was maintained at approximately 10 to 30 copies per cell.

The plasmid pJC104, prepared as described above, was transformed into E. coli K-12 strain XL-1 (recAl, endA1, gyrA96, thl-1, hsdR17, suDEi4, relA1, lac-/F', proAB, lacI^{q}ZdeltaM15,

TN10) cells made competent by the calcium chloride method. In this construction the expression of the rp41 HIV-2 fusion protein was under the control of the lac promoter. Recombinant p41 HIV-2 expression was induced by the addition of IPTG to 100μg/ml. This plasmid replicated as an independent element, wzs non-mobilizable and was maintained at approximately 10 to 30 copies per cell.

The plasmid pTB210, prepared as described above, was transformed into E. coli K-12 strain XL-1 (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac-/F', proAB, lacI^{q}ZdeltaM15, TN10) cells made competent by the calcium chloride method. In this construction the expression of CKS protein was under the control of the lac promoter. CKS expression was induced by the addition of IPTG to 100μg/ml. This plasmid replicated as an independent element, was non-mobilizable and was maintained at approximately 10 to 30 copies per cell.

Example 10

HIV-1/HIV-2 Antibody Assay Using One Solid Phase

One solid phase was coated with the recombinant antigens rp24, rp41, and rp36 as described in Example 3 and used in an assay to detect the presence of HIV-1/HIV-2 antibody in a test sample. The assay conditions were essentially as described in Example 3, except that sample volume was decreased to 50 μL, from 150 μL used in the licensed assay of Example 3. Evaluation was conducted on 121 diluted HIV-2 samples derived frp, 30 individual, a fresh random donor population consisting of 2194 plasma and 980 serum, and 153 HIV-1 seroconversion samples derived from 19 individuals.

Assays using the synthetic HIV-2 then were compared to the results obtained for the same serum sample when using an HIV antibody assay (Human Immunodeficiency Virus Types 1 and 2: E. coli and B. megaterium, recombinant antigen, Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA; available from Abbott Laboratories, Abbott Park, IL), Abbott List 3A77 or Abbott LIst 3A10 following manufacturer's directions as provided in each product insert. The data from these assays are presented in Table 3. The absorbance

of each well was read at 490 nm with a 630 nm reference. The cutoff value of .025 OD + mean OD of the negative control was established. Samples were considered reactive (positive) when the sample/cutoff value was greater than 1 In Table 3, "OD" refers to the optical density reading, "S/CO" means sample/cut-off value, "Result." refers to the interpretation of the test, "NC" refers to negative control and "PC" refers to the positive control.

TABLE 3
HIV-2 DILUTIONAL SENSTIVITY

Assay samples	Number of samples detected No. Positive/Total No.	Mean S/CO HIV-2
3A77	50/121	1.87
rDNA/synthetic HIV-2	106/121	8.62
3A10	102/121	7.73

Individual test samples results which were summarized in Table 3 are given in Table 4, below.

TABLE 4
HIV-2 DILUTIONAL SENSTIVITY*

Sample ID (mean value)	3A77 CO=0.109 S/CO	rDNA/Synthetic HIV-2 CO=0.152 S/CO	3A10 CO=0.113 S/CO
261595	0.52	6.06	3.47
412378	2.59	8.16	7.98
G199	0.63	4.82	4.15
60415K	1.80	10.99	8.57
24310	1.25	7.77	6.48
22804S	1.52	7.94	7.49
25017	3.93	5.40	9.38
G186	0.55	8.36	5.27
G100	1.51	13.17	10.47
25383	0.67	6.26	5.09
39972	1.91	13.45	12.24
G268	0.84	7.74	5.54
22681	0.95	5.52	5.41
22124	1.38	9.55	9.89
18651	1.99	11.11	9.29
20946	1.31	9.35	7.48
30248	0.51	7.91	4.47
011443	2.63	10.69	10.51
18332	1.34	8.81	8.47
30533	1.24	10.96	7.48
30248	0.66	8.59	5.35
4371	10.59	12.49	14.23

41397	0.33	2.57	1.66
24436S	2.10	7.58	8.43
35127	3.92	8.60	10.91
20101	1.11	6.66	6.17
32200	1.41	7.40	6.28
25469	2.06	8.90	10.55
N DANFU	2.77	11.54	11.05
PVINEUL	0.98	11.11	7.40

*Dilution means of 4 samples: 1:5, 1:25, 1:1.125, and 1:625

This data illustrates that the improved HIV-1/HIV-2 rDNA/synthetic HIV-2 peptide offers increased HIV-2 senstivity and improved specificty, without compromising HIV-1 seroconversion sensitivity (Table 5, below). A reduction in the required sample volume will allow use of the assay in settings where 150μl is too restrictive.

TABLE 5
HIV-1 SEROCONVERSION SENSITIVITY USING 50 μL SAMPLE VOLUME

Assay	No. of Samples Detected No. Positive/Total No.	Mean S/CO Seroconversion Samples
3A77	170/249	5.10
3A10	170/249	3.36
rDNA/synthetic HIV-2	172/249	7.19

Example 11

Comparison of the rDNA/synthetic HIV-2 peptide format with A310

Assays were conducted as described in Example 10 using 50 μL sample volumes. A homologous format was used. The results are presented in Table 6, below. The results show a significant decrease in the number of false positives in that assay using the synthetic HIV-2 antigen as a component of the indicator reagent in contrast to a purely recombinant HIV-2 gp36 antigen as indicator.

TABLE 6
SENSITIVITY COMPARISON BETWEEN 3A10 AND rDNA/SYNTHETIC HIV-2 PEPTIDE

Sample ID	rDNA/Synthetic HIV-2 CO=0.152 S/CO	Result	3A10 CO=0.113 S/CO	Result
513	0.86	--	4.31	+

524	2.00	+	17.60	+
525	0.40	--	3.05	+
526	0.40	--	6.98	+
527	1.82	+	17.60	+
528	3.76	+	17.60	+
529	4.29	+	17.60	+
530	0.53	--	8.62	+
531	0.53	--	2.90	+
532	0.49	--	3.03	+
533	0.40	--	2.68	+
534	1.61	+	3,49	+
535	0.40	--	1.34	+
536	0.63	--	6.14	+

It is contemplated that the assay of the invention can be optimized even further by varying assay conditions and/or incubation times, using various combinations of antigen or antibody capture or probe reagents, and other methods, reagents and conditions known to those skilled in the art. Thus, various other antibody capture reagents can be used, including HIV p24, gp120, gp160, p17, and others. The variance of the antibody capture reagent may then require the use of a different antigen capture reagent. All these variations are contemplated to be within the scope of this invention. Also, while some of the assays described in the examples used an automated system, it is well within the scope of the present invention that manual methods or other automated

analyzers can be used or adapted to the assay of the present invention. Therefore, the present invention is meant to be limited only by the appended claims.

WHAT IS CLAIMED IS:

1. An assay to detect the presence or amount of antibodies to HIV-1 and/or HIV-2 in a test sample comprising the steps:

a. contacting the test sample with

i. a first capture reagent attached to a solid phase, wherein said capture reagent is a polypeptide comprising HIV-2 env,

ii. a second capture reagent attached to a solid phase, wherein said capture reagent is a polypeptide comprising HIV-1 env, and

iii. a third capture reagent attached to a solid phase, wherein said capture reagent is a polypeptide comprising HIV-1 gag;

to form a first mixture;

b. contacting saidfirst reaction mixture with

i. a first indicator reagent comprising a synthetic HIV-2 env antigen labelled with a signal generating compound,

ii. a second indicator reagent comprising a HIV-1 env antigen labelled with a signal generating compound, and

iii. a third indicator reagent comprising a HIV gag antigen labelled with a signal generating compound,

to form a second reaction mixture;

c. determining the presence of said HIV-1 env antibodies, HIV-2 env antibodies, and/or HIV gag antibodies in the test sample by detecting the total signal generated by HIVantibody/capture reagent/indicator reagent complexes.

2. The assay of claim 1 wherein said first indicator reagent comprises a synthetic site-directed HIV env antigen having an immunoreactive specificity characteristic of one immunodominant region of gp36 of HIV-2.

3. The assay of claim 2 wherein said immunodominant region of said synthetic HIV antigen is about 120 amino acids from the N-terminal region of gp36 of HIV-2.

4. The assay of claim 2 wherein said synthetic HIV antigen comprises at least one cysteine residue.

5. The assay of claim 2 wherein said synthetic HIV antigen is a peptide selected from the group comprising:

(a)Lys-Asp-Gln-Ala-Gln-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr;

(b) Arg-Val-thr-Ala-Ile-Glu-Lys-Tyr-LeuLys-Asp-Gln-Ala-Gln-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr; and

(c) Val-thr-Ala-Ile-Glu-Lys-Tyr-Leu Glu-Asp-Gln-Ala-Arg-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys.

6. The assay of claim 1 wherein said second indicator reagent comprises a recombinant or synthetic HIV env antigen having an immunoreactive specificity characteristic of the immunodominant region of gp41 of HIV-1.

7. The assay of claim 1 wherein said third indicator reagent comprises a synthetic or recombinant HIV gag antigen having an immunoreactive specificity characteristic of the immunodominant region of p24 of HIV-1.

8. The assay of claim 1 wherein said first, second, and third capture reagents are bound to at least one solid phase.

9. The assay of claim 8 wherein said solid phase is selected from the group consisting of magnetic beads, non-magnetic beads, wells of a reaction tray, microparticles, nylon strips and nitrocellulose strips.

10. The assay of claim 1, wherein said first HIV-1 gp41 env capture reagent, said second HIV-2 gp36 env capture reagent, and said third HIV p24 gag capture reagent are recombinantly or synthetically produced.

compound of the HIV antibody indicator reagent is selected from the group consisting of enzymes, luminescent compounds, chemiluminescent compounds and radioactive elements.

12. The assay of claim 11 wherein said enzyme is horseradish peroxidase.

13. The assay of claim 1 further comprising an indicator reagent comprising a recombinant HIV-2 env antigen labelled with a signal generating compound, said indicator reagent present from 0.014-1.4 micrograms per assay test.

14. A test kit for simultaneously detecting the presence or amount of antibodies to HIV-1 and HIV-2 in a test sample comprising:

(a) a HIV-1 antigen capture reagent comprising at least one polypeptide having an immunoreactivity specificity characteristic of the gp 41 env region of HIV-1 attached to a solid phase;

(b) a HIV-2 antigen capture reagent comprising at least one polypeptide having an immunoreactivity specificity characteristic of the gp 36 env region of HIV-2 attached to a solid phase;

(c) a HIV antigen capture reagent comprising at least one polypeptide having an immunoreactivity specificity characteristic of the p24 env region of HIV attached to a solid phase;

(d) an indicator reagent comprising a synthetic HIV-2 gp36 env antigen labelled with a signal generating compound;

(e) an indicator reagent comprising a HIV-1 gp41 env antigen labelled with a signal generating compound;and

(f) an indicator reagent comprising a HIV gag antigen labelled with a signal generating compound.

15. The test kit of claim 14 further comprising an indicator reagent comprising a recombinant HIV-2 env antigen labelled with a signal generating compound.